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TECHNICAL REPORT

EATR 4644

THE REACTION OF *TRIS*-(CHOLINE CHLORIDE) PHOSPHATE WITH EEL CHOLINESTERASE

by

Joseph W. Amshey, Jr., SP4
George M. Steinberg, Ph.D.

June 1972

DEPARTMENT OF THE ARMY
EDGEWOOD ARSENAL
Biomedical Laboratory
Edgewood Arsenal, Maryland 21010

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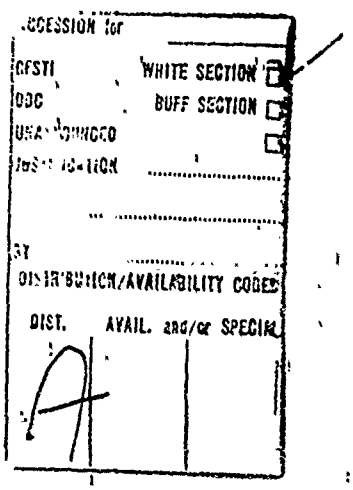

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Medical Research Division

June 1972

Task 1W662710AD2502

DEPARTMENT OF THE ARMY
EDGEWOOD ARSENAL
Biomedical Laboratory
Edgewood Arsenal, Maryland 21010

FOREWORD

The work described in this report was authorized under Task 1W662710AD2502, Medical Defense Against Chemical Agents, Prophylaxis and Therapy for Lethal Agents. This work was started in November 1970 and completed in December 1971. Experimental data are contained in notebooks MN-2463, 2464, 2465, and 2466.

DIGEST

Tris-(choline chloride)phosphate (TCCP) has been observed to slowly and progressively inhibit eel acetylcholinesterase. The kinetics of the inhibition reaction are consistent with a mechanism involving binding of the inhibitor to the enzyme, followed by a rate-limiting irreversible reaction producing inactive enzyme. The dissociation constant of the enzyme-inhibitor complex and the rate constant of the inactivation reaction have been determined to be 0.036 *M* and 0.2 min^{-1} respectively. The quaternary reversible inhibitor tetraethylammonium bromide competes with TCCP for the reaction site. Activation of the enzyme by TCCP toward the hydrolysis of acetylcholine was observed at low ionic strength, but the effect is attributed to the ionic strength contribution of TCCP itself. TCCP does not noticeably reduce the inhibition of enzyme by decamethonium bromide as does the structurally analogous compound flaxedil. The results obtained with TCCP are compared with available data on the relative enzymic reactivity of alkoxy and thiolo esters of phosphoric acid.

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13. ABSTRACT

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14. KEYWORDS

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I. INTRODUCTION.

Triesters of phosphoric acid were first observed to be inhibitors of cholinesterases (E.C. 3.1.1.7 and E.C. 3.1.1.8) by Fukuto,[1] who observed inhibition by 0,0-diethyl 0-(3,3-dimethyl-1-butyl)phosphate. They were also noted by Tammelin[2] while repeating earlier work by Koelle and Steiner,[3] who investigated inhibition of cholinesterases by dialkoxyphosphorylthiocholines and analogues. Tammelin[2,4] obtained pI50 values for several diethylphosphorothiolates and their oxygen analogues. In each case, the sulfur-containing compound was a considerably more potent inhibitor. More recently, O'Brien and coworkers[5-7] extended these studies to a large number of paired phosphates and phosphorothiolates, which they compared in terms of I50 and second-order reaction rate constants, k_i (equation 8). They found that with neutral compounds, the thiolates react more rapidly, but in most cases the differences are quite small (one order of magnitude or less). Bracha and O'Brien[6] attributed these small differences to the general superiority of thiols as leaving groups.*

However, the enhancement in rate caused by the presence of an S instead of the O in the corresponding phosphate esters,[6] for which they coined the term "thiolo effect," is not always small. Two categories of deviations from the general pattern of thiolo effects can be noted, although the small number of examples in each category makes this generalization limited. We will refer to these deviations as abnormal thiolo effects.

With paired families of neutral phosphorus esters, $(EtO)_2P(O)X(CH_2)_n-CH(C_2H_5)_2$ and $(EtO)_2P(O)X(CH_2)_nCH_3$, differences in inhibition rate between X = S and X = O where $n = 2$ or more are small and can be attributed to leaving group effects. The first category of deviants consists of these neutral esters when $n = 1$ or 0. Here the rate ratios are greater by several orders of magnitude.[5,6] In the second category are the basic compounds, including $(EtO)_2P(O)XCH_2CH_2N(C_2H_5)_2$ and *N*-(2-fluoroethyl)-*N*-ethyl and *N*,*N*-di(2-fluoroethyl) analogues.[5-7] Here the rate ratios are even higher, reaching a value of 10.[6] Such differences are entirely too large to attribute to leaving group effects and hence must be enzymic in origin.

Whether the abnormal thiolo effect is a result of an unfavorable binding interaction between alkoxy inhibitor and enzyme or of a reduced rate of phosphorylation can be determined only by separating these two components of the second-order rate constant. Little data of this nature are available. O'Brien and his coworkers[5,6] examined several phosphorothiolates and reported their dissociation constants from bovine erythrocyte cholinesterase and also the rate constants for the irreversible inhibition step. Unfortunately, comparable data were not obtained for the oxygen analogues.

While examining the compound *tris*-(choline chloride)phosphate (TCCP), $P(O)[OCH_2-CH_2\overset{+}{N}(CH_3)_3]_3 \cdot 3Cl^-$, slow irreversible inhibition of eel acetylcholinesterase (AChE) was noted. Because it was convenient to determine binding and phosphorylation rate constants, they were

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[1] Fukuto, T. R. Advan. Pest Control Res. *1*, 147 (1957).

[2] Tammelin, L. E. Acta Chem. Scand. *11*, 1340 (1957).

[3] Koelle, G. B., and Steiner, E. C. J. Pharmacol. Exp. Therap. *118*, 420 (1956).

[4] Tammelin, L. E. Arkiv Kemi *12*, 287 (1958).

[5] Aharoni, A. H., and O'Brien, R. D. Biochemistry *7*, 1538 (1968).

[6] Bracha, P., and O'Brien, R. D. Biochemistry *7*, 1545, 1555 (1968).

[7] O'Brien, R. D., and Hilton, D. B. J. Agr. Food Chem. *12*, 53 (1964).

*Tammelin[4] reported a relative rate factor of five times for reaction of hydroxide ion with a phosphate and its isosteric phosphorothiolate. The latter is the more rapid reactant.

obtained. It was our hope that this information would contribute to our understanding of why some phosphates are unexpectedly poor inhibitors of AChE as K_p and k_p (see equation 1) had not previously been separated for a nonsulfur-containing phosphate.

II. KINETIC SCHEME.

The irreversible inactivation of enzyme by inhibitor can be described by equations (1) and (2) adapted from Main[8]

$$E + PX \underset{}{\overset{K_p}{\rightleftharpoons}} E \cdot PX \xrightarrow{k_p} E' + X \qquad (1)$$

$$E + A \overset{K_A}{\rightleftharpoons} E \cdot A \qquad (2)$$

where E represents enzyme; PX is an irreversible inhibitor with leaving group X, E′ is the phosphorylated enzyme, A is a competitive inhibitor, and K_A and K_P are dissociation constants for the enzyme complexes, which are assumed to be in equilibrium with their precursors. In the equations developed below, the concentrations of the species PX, E · PX, and E · A are given respectively as [P], [EP], and [EA]. Because enzyme is conserved, we have

$$[E]_0 - [E'] = [E] + [EA] + [EP] \qquad (3)$$

which can be converted by substitution of the equilibrium expressions to

$$[E]_0 - [E'] = [EP]\left\{1 + \frac{K_p}{[P]}\left[1 + \frac{[A]}{K_A}\right]\right\} \qquad (4)$$

The reaction can be followed by observing the activity of enzyme, which is proportional to the remaining active enzyme concentration at time t; hence

$$\frac{d([E]_0 - [E'])}{dt} = -k_p\,[EP] \qquad (5)$$

Substitution of equation (4) into (5) and integration gives the expression

$$R = \frac{\Delta \ln v}{\Delta t} = \frac{\ln([E]_0 - [E'])_2 - \ln([E]_0 - [E'])_1}{t_2 - t_1} = \frac{-k_p}{1 + \frac{K_p}{[P]}\left(1 + \frac{[A]}{K_A}\right)} \qquad (6)$$

Inversion of equation (6) predicts that a linear double-reciprocal plot (equation 7) should be

$$\frac{1}{R} = \frac{K_p}{k_p}\left(1 + \frac{[A]}{K_A}\right) \cdot \frac{1}{[P]} + \frac{1}{k_p} \qquad (7)$$

obtained for $1/R$ versus $1/[P]$, with its slope equal to the quantity $(1 + [A]/K_A)K_p/k_p$. The intercept on the ordinate is equal to $1/k_p$. When [A] = 0, the slope will be K_p/k_p, allowing the separation of rate and equilibrium constants.

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[8]Main, A. R. Science *144*, 992 (1964).

in the absence of A, from equation (6), the second-order reaction rate constant, k_i, is

$$k_i = \frac{R}{[P]} = \frac{k_p}{[P] + K_p} \tag{8}$$

defined in equation (8). With rapid reactants, $K_p >> [P]$ so that $k_i = k_p/K_p$.

III. EXPERIMENTATION.

A. Materials.

Tris-(choline chloride)phosphate was prepared by Ash-Stevens, Inc., under contract DAAA15-69-C-0584, according to the procedure described by Jackson.[9] Analysis observed: 38.90% C, 8.60% H, 9.06% N; analysis calculated: 38.92% C, 8.49% H, 9.08% N. Tetraethylammonium bromide (TEA) was a recrystallized Eastman product obtained from Dr. J. C. Kellet, Jr. The hydroscopic salt was dried at 110°C, and then stored over P_2O_5. Phenyl acetate was an Eastman product. Decamethonium bromide $[(CH_3)_3\overset{+}{N}(CH_2)_{10}\overset{+}{N}(CH_3)_3 \cdot 2Br^-]$ was provided by Dr. E. Bay. Eel acetylcholinesterase (AChE) was purchased from Worthington and stored in stock solutions containing 0.225 *M* KCl, 0.1% gelatin, and 0.02% NaN_3. Acetylcholine chloride (ACh) was obtained in 100-mg preweighed ampoules from Nutritional Biochemicals Company. Stock solutions 0.1101 *M* in ACh were prepared by quantitatively dissolving the contents of one ampoule so as to make 5 ml of solution. Gallamine triethiodide [flaxedil; 1,2,3-*tris*-(2-triethylammonioethoxy) benzene triiodide] was obtained from Davis and Peck and used without further purification.

B. Methods.

Inhibition of the enzyme AChE by TCCP was measured in 0.1 *M* morpholinoethane sulfonic acid (MES) buffer,[10] *p*H 6.61. Stock solutions of TCCP were prepared in MES buffer just prior to use. Inhibition reactions were initiated by adding a small (25 μl) volume of enzyme stock solution at a convenient concentration to an appropriate dilution of TCCP stock with MES. The enzyme concentration range was estimated to be 1.2 to 5×10^{-9} *M*.[11] The ionic strength was held constant at that of 0.066 *M* TCCP by the addition of solid KCl as needed, and 100 μl of TEA stock solution at 33.8 m*M* were added to reactions requiring a competitive inhibitor. The final concentration of TEA in inhibited reactions was 1.05 m*M*. The disappearance of active enzyme was followed by withdrawing 100 μl aliquots at intervals and assaying for enzyme activity in 3 ml of 0.1 *M* MES buffer, *p*H 6.61, containing 8 m*M* phenyl acetate. The hydrolysis of phenyl acetate could be conveniently followed at 270 nm on a Cary 14 recording spectrophotometer.

The observed phenyl acetate hydrolysis rates, *r*, were plotted as *ln r* versus incubation time with TCCP to obtain pseudo-first-order reaction rate constants *R*. First-order plots were linear for more than two half times. These rate constants were plotted in the double-reciprocal form of equation (7) as shown in figure 1 to obtain the dissociation constant K_p and the first-order rate constant k_p.

The activity of chloride ion in TCCP solutions was measured by a Beckman chloride ion electrode with a calomel reference electrode on a Beckman research *p*H meter. A standard curve of electrode potential at 25°C for known chloride concentrations was obtained by diluting stock KCl solutions in glass-distilled deionized water with the ionic strength made up with KNO_3.

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[9] Jackson, E. L. J. Amer. Chem. Soc. *57*, 1903 (1935).

[10] Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S., and Singh, R. M. M. Biochemistry *5*, 467 (1966).

[11] Stemberg, G. M., Berkowitz, L. M., Thomas, N. C., Maddox, J. P., and Szatrapiec, L. L. J. Pharm. Sci. *61*, 527 (1972).

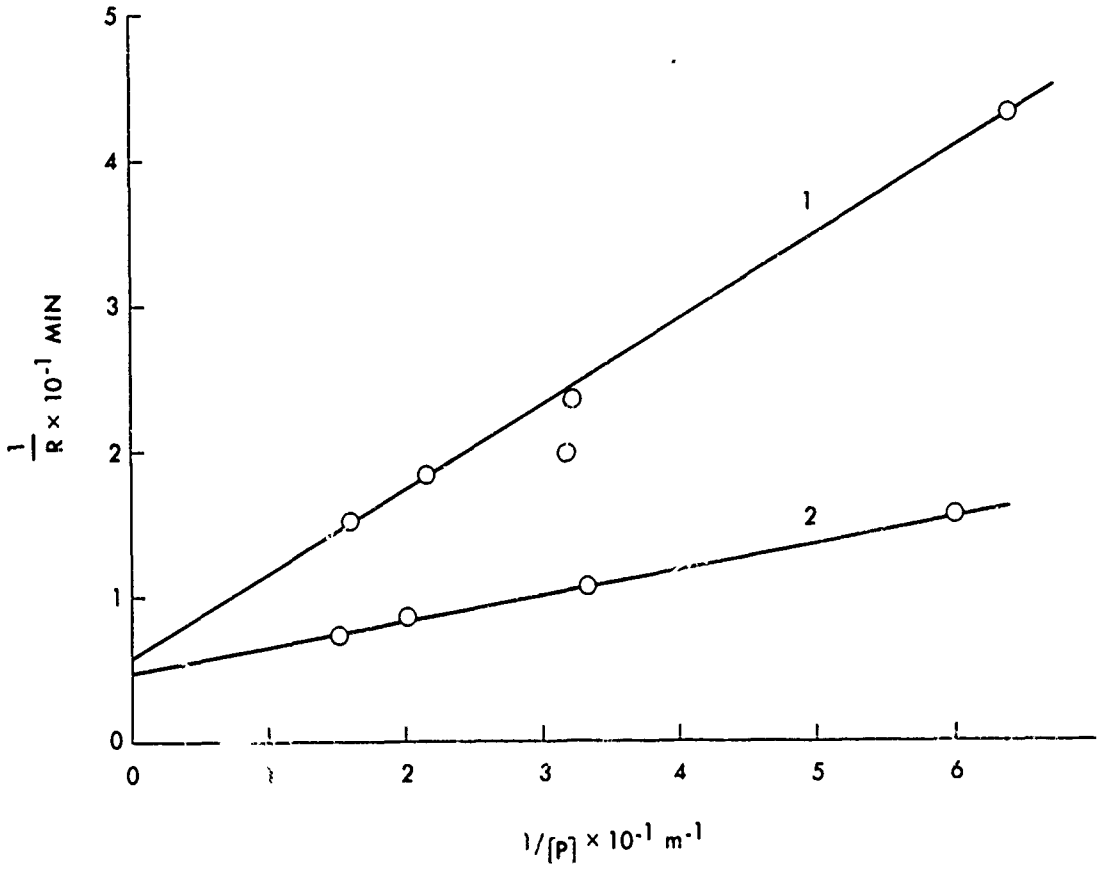

Figure 1. Inhibition of AChE by TCCP, MES Buffer 0.1 *M*, *p*H 6.61. 25°C

Curve 1 reaction contains 1.05 m*M* tetraethylammonium Br.
Curve 2 uninhibited.

Competitive inhibition constants K_I were calculated from observed K_m [$K_{m(obs)}$] values obtained with a Wilkinson[12] weighted regression analysis of reciprocal relationships. Strictly competitive inhibition was observed so that K_I values could be calculated using equation (9).

$$K_{m(obs)} = K_m \left(1 + \frac{[I]}{K_I}\right) \tag{9}$$

Effects of salts and TCCP on AChE activity were measured using a Radiometer *p*H stat. The hydrolysis of 1×10^{-3} *M* acetylcholine was followed in 3.30 ml total volume of solution at *p*H 7.15 by observing the rate of addition of 0.0091 *N* KOH needed to maintain the *p*H of the solution. Salt concentrations were changed by varying the relative amounts of 0.0001 *M* KCl and 1 *M* stock salt solutions.

Partial reversal of decamethonium inhibition of AChE by flaxedil was obtained in 0.003 *M* MES buffer *p*H 6.61 containing 8 m*M* phenyl acetate. Sufficient enzyme was added to a cuvette containing buffer, substrate, and 2×10^{-6} *M* decamethonium bromide to give a slow but measurable hydrolysis of the phenyl acetate as observed at 270 nm on the Cary 14 spectrophotometer. The comparative effects of TCCP and flaxedil were observed by adding them to this system at a concentration of 4×10^{-5} *M*.

[12]Wilkinson, G. N. Biochem. J. *80*, 324 (1961).

IV. RESULTS AND DISCUSSION.

Figure 1 is a double-reciprocal plot according to equation (7). It indicates that the data are consistent with the mechanism assumed in equation (1), and that the inhibitor tetraethylammonium bromide (TEA) is competitive with TCCP. K_A calculated for TEA from the data in figure 1 is 4.8×10^{-4} *M*, in good agreement with a competitive K_I for inhibition of phenyl acetate hydrolysis by TEA of 4.9×10^{-4} *M*. The latter K_I was determined in 0.1 *M* MES buffer, *p*H 6.61, containing 0.357 *M* KCl to adjust the ionic strength to that present in the TCCP phosphorylation reactions. This result suggests that TCCP is binding to the enzyme active site, and presumably this is also the site of its inactivation reaction. From the double-reciprocal plot in the absence of TFA, the value of k_p and K_p can be calculated to be 0.2 min^{-1} and 0.036 *M* respectively. Further, the competitive K_I value for TCCP determined from its inhibition of phenyl acetate hydrolysis is 0.0246 *M*. Its reasonably close agreement with the value of K_p provides additional support for TCCP reaction at the enzyme active site.

It had been suggested by Overberger et al.[13] that TCCP might exist in an ion-pair form so that the actual ionic strength of a solution would be much less than that calculated assuming complete dissociation of the chloride ions. To test this, the concentration of free chloride in a series of TCCP solutions was measured with a chloride ion-sensitive electrode. A plot of observed chloride ion activity versus molar concentration of TCCP from 1 to 5 m*M* has a slope of 3.1, indicating complete dissociation of all chloride from the three quaternary nitrogens as the electrode is insensitive to ion-pairs. The results of this experiment are shown in figure 3.

The activity of acetylcholinesterase toward acetylcholine in increasing concentrations of TCCP was examined for unusual effects. Figure 2 is a comparison of the activating properties of TCCP, K_2SO_4, and $MgCl_2$ plotted as a function of ionic strength. Because of the structural similarity between TCCP and gallamine triethiodide (flaxedil), TCCP was examined for the ability to reverse the inhibition of eel AChE by decamethonium, which can be seen with flaxedil at low ionic strengths.[14] At concentrations of TCCP comparable to those at which we observed that flaxedil decreased the extent of inhibition produced by decamethonium by 3.94-fold, the effect of TCCP was only 1.07-fold, an amount that easily can be attributed to the ionic strength contribution of the salt.

In table I are listed K_p, k_p, and k_i values for a series of dialkylphosphorothiolates, k_i values for related phosphates together with the values obtained in this work for TCCP. It is striking that TCCP, in spite of its three positive charges and choline leaving group, binds so very poorly to AChE. Like the other phosphates, V and VIII, its k_i value is also quite low, being perhaps three to six orders of magnitude smaller than those of the rapid phosphorylators. In table II, comparisons are given of the values of K_p, k_p, and k_i for TCCP and four of the phosphorothiolates listed in table I. The marked reduction in k_i for TCCP cannot be assigned exclusively either to K_p or k_p. Diminished reaction is a result of decrements in both parameters.

The cause of the abnormal thiolo effect (at least in the nitrogen-containing phosphates) would appear to be neither strong but misoriented binding nor rapid reaction but very poor binding. The effect is not a result of appreciable differences in extent of protonation of the amino groups at neutral *p*H in paired compounds such as II and V. Both are extensively protonated because their pK_a values are close to 8.[5] Neither is the difference exclusively a result of the positive charge on the nitrogen atom. Fluorinated derivatives of II and V, the *N*-(2-fluoroethyl)-*N*-ethyl and

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[13]Overberger, C. G., et al. University of Michigan. Semiannual Contract Report, January-June 1969. Contract DAAA-15-67-C-0567. Mechanism of Enzyme Action. UNCLASSIFIED Report.

[14]Changeux, J. P. Mol. Pharmacol. *2*, 369 (1966).

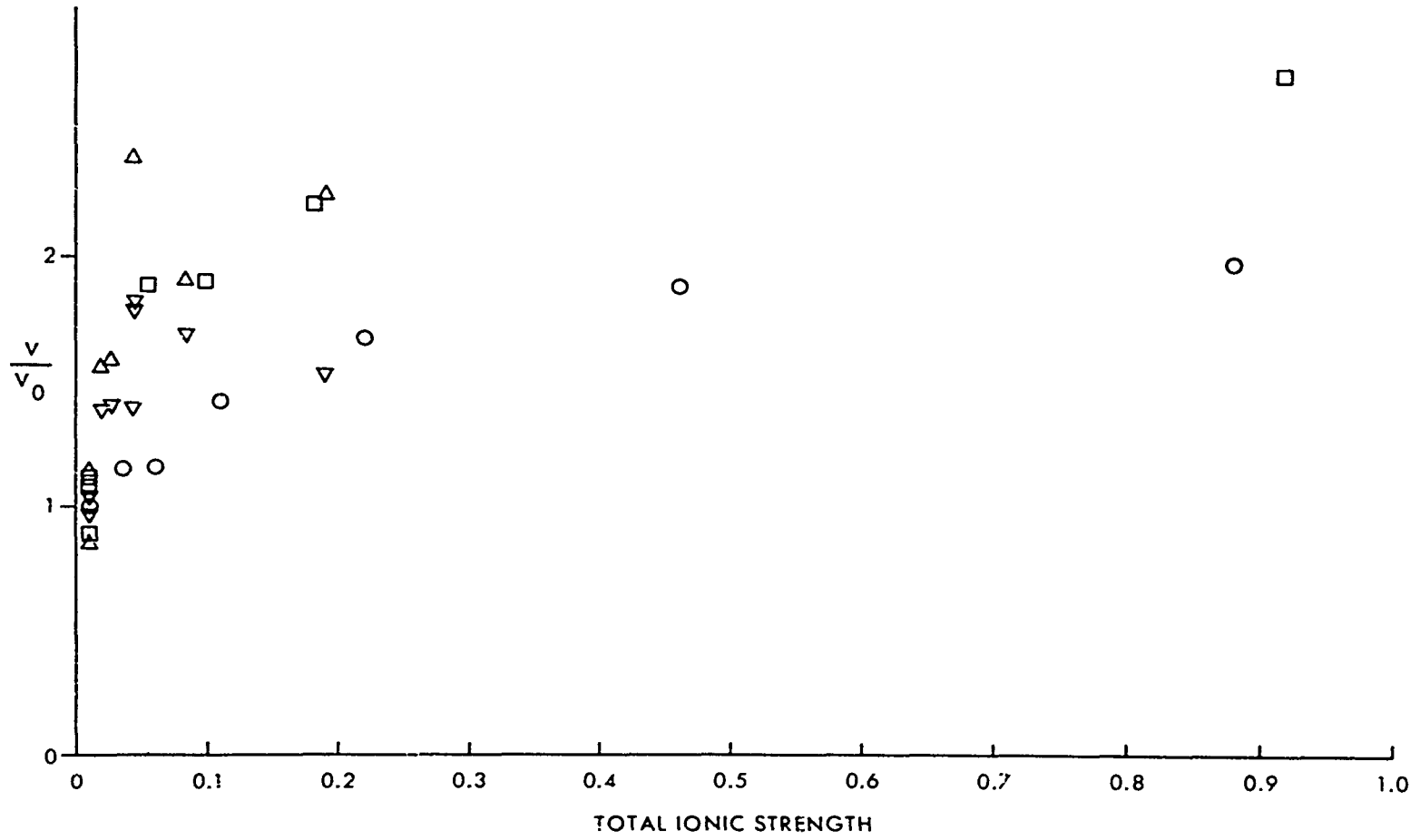

Figure 2. Activation of AChE by Increased Ionic Strength

□ $MgCl_2$, *p*H 7.15
△ TCCP, *p*H 6.20
○ K_2SO_4, *p*H 7.15
▽ TCCP, *p*H 7.15

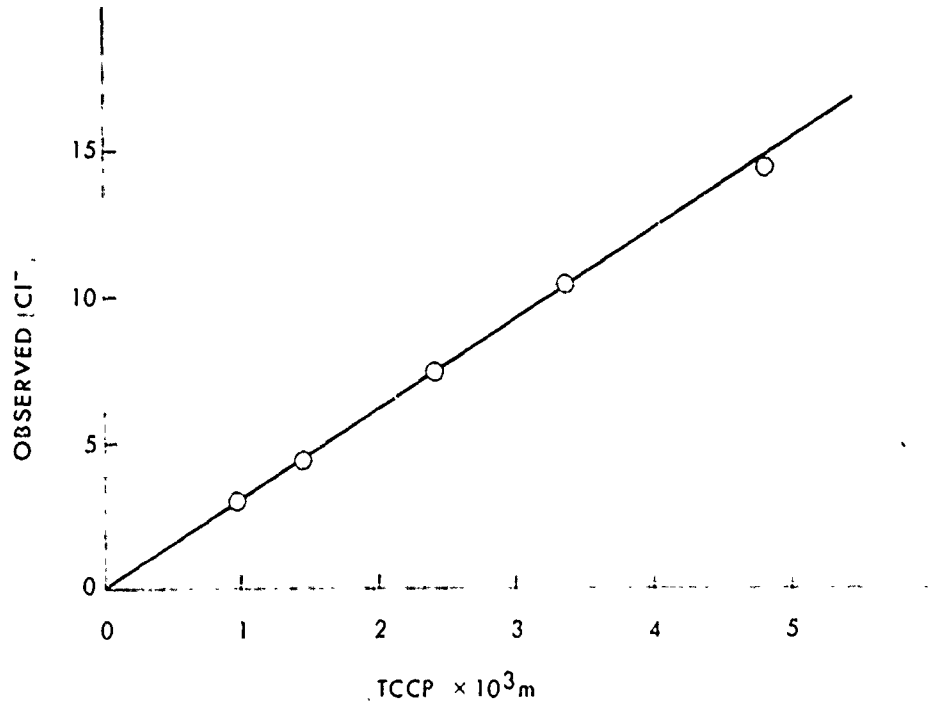

Figure 3. Dependence of Chloride Ion Concentration on TCCP Concentration

N,N-di-(2-fluoroethyl) analogues, are unprotonated at the reaction *p*H; yet, the abnormal thiolo effect appears[7] (in spite of their isosteric relationships to I and VII, both of which react very rapidly with AChE). Finally, abnormal thiolo effects are not caused by appreciable differences in their nucleophilic reactivity.[4] Thus, we find no simple unifying principle to include all the observed results with the phosphates.

With acetyl esters, significant differences exist between some oxygen and sulfur (thiolo) esters. Whereas Hillman and Mautner[15] have observed that acetylcholine and acetylthiocholine are very similar in turnover rate, Augustinsson and Isachen[16] reported that there was a marked difference between the corresponding β-methylcholine and β-methylthiocholine esters. For the oxygen ester (mecholyl), the D isomer hydrolyzes much faster than the L isomer so that hydrolysis "stops" when one-half of the ester has been consumed. With the analogous thiol ester, all of the ester (both isomers) is hydrolyzed by AChE in one rapid continuous step. Combined, these observations suggest the generalization that with thiol esters there is greater accommodation of the enzyme to adverse structural features. When the structures of the reacting paired (O and S) molecules do not contain adverse structural features, reaction rates with AChE are closely similar. When fit becomes strained, the rate of reaction with the oxygen analog falls off, whereas the rate of reaction of the sulfur analog remains at the normal elevated level.

[15] Hillman, G. R., and Mautner, H. G. Biochemistry *9*, 2633 (1970).
[16] Augustinsson, K. B., and Isachen, T. Acta Chem. Scand. *11*, 750 (1957)

Table I. Kinetic Constants for Reaction With Acetylcholinesterase

Compound	K_p	k_p	k_i
	M	min^{-1}	$M^{-1}\ min^{-1}$
I $(C_2H_5O)_2P(O)SCH_2CH_2CH(C_2H_5)_2$	4.48×10^{-5}[a] 6.16×10^{-5}[b]	4.49 2.58	2.3×10^5 4.2×10^4
II $(C_2H_5O)_2P(O)SCH_2CH_2N(C_2H_5)_2$	7.16×10^{-6}[a] 2.8×10^{-4}[c]	6.66 157	1.4×10^6 5.6×10^5
III $(CH_3O)_2P(O)SCH(CO_2C_2H_5)CO_2C_2H_5$	7.7×10^{-4}[b]	11	1.4×10^4
IV $(C_2H_5O)_2P(O)SCH_2CH_2\overset{+}{N}(C_2H_5)_3$	2.48×10^{-5}[a,d]	115	6.9×10^6
V $(C_2H_5O)_2P(O)OCH_2CH_2N(C_2H_5)_2$	–[a]	–	1.98
VI $(C_2H_5O)_2P(O)SCH(CH_2N(CH_3)_2)_2$	1.2×10^{-4}[a]	55	4.1×10^5
VII $(C_2H_5O)_2P(O)OCH_2CH_2CH(C_2H_5)_2$	–[e]	–	7.3×10^4
VIII $(C_2H_5O)_2P(O)OCH_2CH_3$	–[f]	–	0.42
TCCP, $P(O)(OCH_2CH_2\overset{+}{N}(CH_3)_3)_3$	3.6×10^{-2}[g]	0.2	5.56

[a] *p*H 7.4, phosphate, RBC AChE; K_p and k_p at 25°C; k_i at 38°C. *From* Aharoni, A. H., and O'Brien, R. D. Biochemistry 7, 1538 (1968).[5]

[b] *p*H 7.4, phosphate, RBC AChE, 25°C. *From* Bracha, P., and O'Brien, R. D. Biochemistry 7, 1545, 1555 (1968).[6]

[c] *p*H 7.0, RBC AChE, 5°C. *From* Main, A. R. J. Biol. Chem. *244*, 829 (1969).[17]

[d] The values K_p and k_p as reported are open to some question because of the considerable error observed in their measurement.

[e] Kabachnik, M. I., Brestkin, A. P., Godovikov, N. N., Michelson, M. J., Rozengart, E. V., and Rozengart, V. I. Pharmacol. Rev. *22*, 355 (1970).[18] Calcd. from data *in* Bracha and O'Brien, *op. cit.*

[f] Rat brain AChE, 25°C. *From* Gumbmann, M. R., and Williams, S. N. J. Agr. Food Chem. *18*, 76 (1970).[19]

[g] This work, *p*H 6.61, eel AChE, 25°C. The value of k_i here has been set equal to k_p/K_p; see eq 8 and accompanying discussion.

[17] Main, A. R. J. Biol. Chem. *244*, 829 (1969).

[18] Kabachnik, M. I., Brestkin, A. P., Godovikov, N. N., Michelson, M. J., Rozengart, E. V., and Rozengart, V. I. Pharmacol. Rev. *22*, 355 (1970).

[19] Gumbmann, M. R., and Williams, S. N. J. Agr. Food Chem. *18*, 76 (1970).

Table II. Ratios of Kinetic Constants of TCCP to Those of Several Dialkylphosphorothiolates

Compound[a]	K_p (TCCP/Cpd)	k_p (Cpd/TCCP)	k_i[b] (Cpd/TCCP)
	M	min^{-1}	$M^{-1}\ min^{-1}$
I[c]	580	13	7.6×10^3
II[d]	5000	33	2.5×10^5
III[c]	47	55	2.5×10^3
VI[c]	300	275	7.4×10^4

[a]Structures are given in table I.
[b]Calculated from values in table I.
[c]*From* Bracha and O'Brien.[6]
[d]*From* Aharoni and O'Brien.[5]

The reason for the apparent adaptability of the enzyme with sulfur esters is not known. It could be related to sulfur's larger covalent radius, somewhat different bond angles, lower electronegativity (and reduced ability to form hydrogen bonds), or its greater polarizability. Based upon the well-known evidence for substrate-induced conformational changes in enzymes as a result of their interaction in the Michaelis complex,[20] we are inclined to view abnormal thiolo effects in these terms. If one were to suppose greater interaction (hydrogen bonding) between the substrate oxygen atom and a proton at or very close to the esteractic site, it could well result in greater constriction in the enzyme conformation. Such restricted conformation, with its reduced flexibility, would be compatible with increased specificity the reduced ability to accommodate to diverse structures.

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